BIODIVERSITY AND PHENOTYPIC CHARACTERIZATION OF SACCHAROMYCES CEREVISIAE STRAINS ISOLATED FROM THE VINHO VERDE WINE REGION

D. Schuller, H. Alves, P. Sampaio, C. Pais and M. Casal

Centro de Biologia, Universidade do Minho, 4710-057 Braga, Portugal

Margarida Casal
Centro de Biologia
Campus de Gualtar
Universidade do Minho
4710-057 Braga, Portugal
Tel: 253 - 60 40 44
Fax: 253 – 67 89 80
Mail: mcasal@bio.uminho.pt

Introduction

The grape's yeast flora depends on a large variety of factors such as climatic conditions including temperature and rainfalls, the geographic localization of the vineyard, antifungal applications, the harvest technique, grape variety, the vineyard's age as well as the soil type. Several ecological surveys report a large diversity of Saccharomyces sp. strains among the enological fermentative flora. Some strains seem to be widely distributed in a given viticultural region, can be found in several consecutive years and are also predominant in the fermenting flora hypothesizing the occurrence of specific native strains that can be associated to a terroir.

At present, leading winemakers demand for autochthonous fermenting strains that are able to enhance the expression of typical sensorial characteristics of wine and ensure the control of the fermentation process, concerning the motto "special yeasts for special traits". The detailed biogeographical evaluation of fermentative strains is essential for the establishment of adequate selection programes.

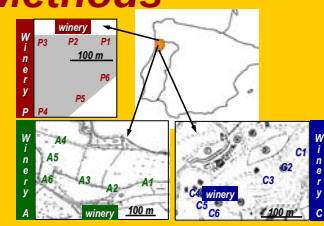
Wines produced in the Vinho Verde region, located in the North of Portugal, are characterized by their fruity and sparkling aroma. A frequent problem is their high volatile acidity, being often above 1.2, the limit established by national regulations. The application of specialized fermentative strains, efficiently utilizing acetic and malic acid, may play a key role in the solution of this inconvenient.

The present study is a systematical, 2-years survey aiming at the characterization of the existing biodiversity and special phenotypical traits, and is the first biogeographical survey of fermentative strains using microsatellites for the analysis of the genetic variability among and within populational structures.

Materials and Methods

Samples

The sampling plan included 18 sites in 3 vineyards, located in the North of Portugal (Região Demarcada dos Vinhos Verdes), as shown. In each vineyard, six sampling points were defined. A first sampling campaign was performed (early stage) some days before the harvest. Some days after the harvest grapes were collected in a second sampling campaign (late stage). This experiment was repeated in two consecutive years (2001-2002).

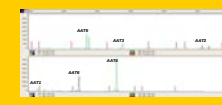


Fermentation

The yeast flora from fermenting grape juice (500 ml) was analysed when the must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content. Must samples were diluted and spread on plates with YPD medium and 30 randomly selected colonies were collected from each spontaneous fermentation and subjected to analysis.

DNA isolation

Yeast cells were cultivated in 5 ml of YPD medium (24 h, 28°C, 160 rpm) and DNA isolation was performed using the method described by López et al. [1].



Microsatellite amplification

The six trinucleotide microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5 and ScAAT6 were amplified [3]. Samples were separated in the ABI Prism 310 DNA sequencer (Applied Biosystems) and analyzed with the corresponding GENESCAN software. The equivalence of this typing method to previously described ones has been shown for the case of commercial S. cerevisiae strains [4] (see also poster E13).

Phenotypic characterization

The ability to sustain growth on media containing ethanol and acetic acid was tested on YNB medium (Difco) containing glucose (2.0%, w/v), acetic acid (0.25%, v/v) and ethanol (10.0%, v/v), adjusted to pH 4.0.

The capacity to utilize malic or acetic acid was investigated on YP medium, pH 4.0, containing methyl orange (0.005%, w/v) and acetic acid (0.25%, v/v) or malic acid (0.5%, w/v). Hydrogen sulphide production was tested on Biggy agar

Computer assisted data analysis

A similarity matrix of allelic frequencies was computed by the program NTSYSpc 2.0 [5], based on the Euclidean distance and average linkage (UPGMA). NTSYSpc 2.0 was also used to draw the dendrogram. For statistical population genetic analysis AMOVA parameters were computed by the Arlequin software [2].

RESULTS

Winery	Year	Stage	Sampling site	Number of isolates	Number of unique patterns
A	2001	early	No fermentation	-	-
		late	A2 A3 A5	90	11
	2002	early	No fermentation *	-	-
		late	A1 A2 A3 A4 A5 A6	180	34
C	2001	early	C4 C5 C6	90	7
		late	C2 C3 C4 C5 C6	150	29
	2002	early	C4	30	1
		late	*	-	-
P	2001	early	P1 P2	60	3
		late	P1 P2 P3 P4 P5 P6	180	68
	2002	early	No fermentation *	-	-
		late	P1 P2 P3 P4 P5 P6	150	16

(*) Several samples could not be collected due to a very bad sanitation state of the grapes after heavy rainfalls.

Table 1: Strains collected

- The strain collection obtained from this survey comprises 930 isolates, that were classified in 169 unique genetic patterns.
- Only few of the 6 samples collected in the early stage completed a spontaneous fermentation, and merely 11 different genetic patterns were obtained from the 180 isolates.
- 158 patterns and 750 isolates of Saccharomyces sp. strains were collected in the late stage.
- The highest biodiversity was observed in winery P (390 isolates, 87 patterns), followed by winery A (270 isolates, 44 patterns) and C (270 isolates, 37 patterns).
- With a few exceptions, in each winery, the groups of patterns found in the early sampling stage is different from the ones found in the late sampling stage.
- The same was verified for the 2 sampling years 2001 and 2002 (not shown).
- Globally, these findings indicate a high biodiversity of the fermenting flora in the ecosystems surrounding the 3 wineries.

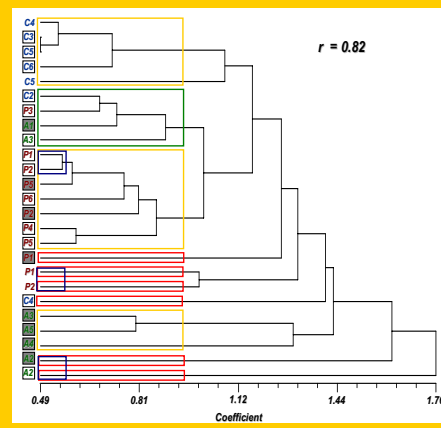


Figure 1: Populational analysis

- Groups of strains were found in the ecosystems of the 3 wineries (yellow squares).
- Strains found in the more distant wineries (around 150 km) are more similar (C and P).
- Occurence of a strain cluster from the 3 wineries (green square), can be explained by the existence of "regional" strains.
- No ubiquitous strain was found and only one common pattern has been found among all strains isolated from winery A and P.
- Some individual populations seem to be completely unrelated to the majority of populations from the same ecosystem (red squares).
- There are distinct populations in the two sampling stages e.g. P1 and P2 or in the 2 sampling years (A2) as indicated by blue squares.
- Globally, the present results speak in favor for the existence of general, regional-wide strains, but at the same time specialized populations, typical for a specific winery. These strains may be subjected to locally limited specific evolutionary processes

Variation	Winery	Grouped samples	Source of variation	Percentage of variation	FST P (r<o)
among 3 wine regions	A–C–P	{A2 A3 A5 A1 A4 A6 P1 P2 P3 P4 P5 P6} {C4 C5 C6 C4 C2 C3 C4 C5 C6} {P1 P2 P1 P2 P3 P4 P5 P6 P1 P2 P3 P4 P5 P6}	AG	4.36	
			APWG	8.47	0.00000
			WP	87.16	
between sampling years	A	{A2 A3 A5} {A1 A2 A3 A4 A5 A6}	AG	-2.06	
			APWG	14.61	0.02933
			WP	87.45	
	C	{C4 C5 C6 C2 C3 C4 C5 C6} {C4}	AG	37.23	
			APWG	3.79	0.31769
			WP	58.98	
	P	{P1 P2 P1 P2 P3 P4 P5 P6} {P1 P2 P3 P4 P5 P6}	AG	0.34	
			APWG	6.68	0.02737
			WP	92.98	
2001 / 2002	C	{C4 C5 C5 C4} {C2 C3 C4 C5 C6}	AG	-1.68	
between sampling time early / late			APWG	9.51	0.29814
			WP	92.17	
	P	{P1 P2} {P1 P2 P3 P4 P5 P6 P1 P2 P3 P5 P6}	AG	5.40	
			APWG	6.01	0.02737
			WP	88.60	
among sampling sites 1/2/3/4/5/6	A	{A1} {A2 A2} {A3 A3} {A4} {A5 A5} {A6}	AG	-7.09	
			APWG	20.23	0.03519
			WP	86.86	
	C	{C2} {C3} {C4 C4 C6} {C5 C5} {C6 C6}	AG	-1.57	
			APWG	10.21	0.27566
			WP	91.37	
	P	{P1 P1 P2} {P2 P2 P2} {P3 P3} {P4} {P5 P5} {P6 P6}	AG	-1.49	
			APWG	8.20	0.03128
			WP	93.29	

AG among groups, refers to the groups of populations in brackets
APWG ampng populations within a group in brackets
WP within populations

Table 2: Population statistical analysis

- AMOVA analysis showed that the only significant differences (p<0.01) were found for the group of strains from each winery.
- No significant differences were found between the samples collected in the different stages of grape maturation and sampling years, for each of the three wineries. The same was verified for the distinct sampling sites.
- For all analysis performed, the highest percentage of variability was always found within the populations corresponding to the sampling sites.

Ethanol resistance	Malic acid utilization	Acetic acid utilization	H_2S production	Number of isolates
-	-	-	+	4
-	-	-	++	2
-	-	+	-	2
-	-	+	+	20
-	-	+	++	10
-	-	++	+	12
-	-	++	++	6
-	+	+	+	6
-	+	+	++	4
-	+	++	+	5
-	++	+	+	1
-	++	++	+	1
+	-	-	+	6
+	-	+	-	5
+	-	+	+	48
+	-	+	++	6
+	-	++	-	2
+	-	++	+	20
+	-	++	++	3
+	+	-	+	1
+	+	-	-	1
+	+	+	+	10
+	+	+	++	5
+	+	++	+	6
++	-	-	+	1
++	-	+	+	7
++	-	+	++	3
++	-	++	-	1
++	-	++	+	6

Table 3 Phenotypic traits

One representant strain from each genetic profile was used for phenotypical analysis to evaluate the strains capability to

- Tolerate high ethanol concentrations in the presence of acetic acid at low pH values,
- Utilize efficiently malic and acetic acid
- Produce a low amount of H_2S.

- A remarkable phenotypical diversity was observed. The most frequently occurring metabolic profiles (red cells) was associated to
  - ► low (-) or good (+) ethanol tolerance in the presence of acetic acid
  - ► intermediate (+) or good (++) capability to utilize acetic acid
  - ► moderate (+) H_2S production
  - ► no capacity to utilize malic acid.
- Only 35 strains were able to utilize malic acid, being two of them very efficient concerning malic acid metabolism.
- The 9 strains marked by a red square are preferential candidates for further studies, the strains marked by a dotted red square are of secondary importance.
- 9 of the 168 strains exhibit interesting phenotypical traits that eventually can have a positive impact as future "specialist strains" for the Vinho Verde Region.

CONCLUSIONS

The vineyard environment is a wine yeast reservoir to select "specialist" strains.

The ecosystems surrounding the 3 wineries in the Vinho Verde Region revealed a considerable biodiversity in fermenting Saccharomyces sp. strains. The appearance of distinct strains at varying sampling times and in consecutive years reinforces the importance of exhaustive sampling procedures in order to obtain representative data. In global terms, the populations from the 3 wineries are distinct, indicating probably the occurence of locally limited evolutionary processes.

The screening of numerous isolates is the unique approach to find rarely occuring natural strains with desired phenotypical traits.

We believe that exploring the biodiversity of indigenous fermentative strains, using simple selection criteria is the basis for further studies that provide deeper insight in the genetic variability. As the use of genetically modified yeasts in winemaking is a highly controversial topic, we consider that the systematic exploration of a wine regions' biodiversity is an important contribution towards the selection and understanding of strains carrying specific enological traits. Such studies are an essential complement to the existing knowledge about genetically modified strains.

Microsatellite typing is an appropriate method for detailed biogeographical surveys.

Strain typing using the six described loci AAT1 – AAT6 gave detailed biogeographical data of winery-associated fermentative Saccharomyces sp. strains, being capable to reveal differences between strains isolated from geograpically close locations.

Strain biodiversity is not significantly affected by the use of commercial yeast strains.

In all wineries commercial starter yeasts were used during at least 7 years. This practice may reduce, and eventually eliminate the biodiversity of autochthonous yeast populations and contribute to the homogeneization of fermentative strains associated to winemaking environments. However, this phenomenon was not verified for the ecosystem surrounding the winery.

References

[1] López V., Querol A., Ramón D., and Fernández-Espinar M.T. 2001. Int J Food Microbiol 68:75-81

[2] Schneider, S., Kueffer, J-M., Roessli, D. and Excoffier, L. 1997. ARLEQUIN version 1.1. Software for population genetics data analysis. Geneva, Switzerland

[3] Pérez, M.A., Gallego, F.J., Martinez, I. and Hidalgo, P. 2001. Lett. Appl. Microbiol. 33, 461-466.

[4] Schuller, D., Valero, E., Dequin, S. and Casal, M. 2003. FEMS Microbiol Lett. in press

[5] NTSYSpc 2.0, 1997, Exeter Software (http://www.ExeterSoftware.com)

Acknowledgements

This study was supported by the grant nº 657 C2 from the cooperation agreement between the Portuguese Institute for International Scientific and Technological Cooperation (ICCTI) and the French Embassy in Lisbon.